Prenatal maternal effects appear to be insensitive to experimental or natural environmental variation

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Abstract

1. In many birds, hatching asynchrony is a common phenomenon, primarily driven by patterns of incubation behaviour. However, experimental results in blue tits (*Cyanistes caeruleus*) have shown that asynchrony is reduced by intrinsic properties of later eggs that accelerate pre-natal development.

2. These intrinsic differences between early and late eggs could be driven by changes in resource availability to females, which are then passively passed onto the egg. Alternatively, it may be due to an anticipatory maternal effect, wherein some signal or resource is actively placed within the egg, which is beneficial to those eggs laid late within the clutch.

3. In order to distinguish between these hypotheses we designed a supplementary feeding experiment, wherein females were provided with food at certain times during the laying phase. This had no discernible effect on development rate, or other egg characteristics, consistent with anticipatory maternal effects.

4. Using a larger data set we also tested whether natural environmental variation (weather) during egg formation affected maternal investment in eggs. Similarly, egg characteristics were found to be relatively insensitive to the environmental variation, supporting the experimental results.

Keywords: anticipatory maternal effect, *Cyanistes caeruleus*, development, eggs, food supplementation, passive effects, weather

Introduction

Parents influence the phenotypes of their offspring, both through the genes they pass on, and by directly modulating the environment offspring experience. These parental

effects have been shown to be present in many systems, and can have a major causal contribution to an individual's phenotype (Roach & Wulff 1987; Mousseau & Dingle 1991; Mousseau & Fox 1998; Badyaev & Uller 2009; Wolf & Wade 2009). The effects are often context-dependent – varying with the environment that the parents themselves experience (Rossiter 1996, 1998). This could occur through the direct response of a parent to changes in their own environment, which are then passed to offspring, regardless of the phenotypic or fitness consequences of these effects to the offspring. Alternatively, parents may use changes in their own environment to anticipate that which their offspring will experience, and actively modulate their offsprings' phenotypes accordingly (Marshall & Uller 2007). Although the context-dependency of parental effects is unequivocal, whether anticipatory responses are a common feature of context-dependency remains contentious (Uller *et al.* 2013; Burgess & Marshall 2014). Anticipatory parental effects (Marshall & Uller 2007) are expected to occur when the environment experienced by parents is a predictor of the environment that will be experienced by the offspring (Burgess & Marshall 2014), and selection therefore favours parents producing offspring of an appropriate phenotype for that environment. Whilst there has been some convincing evidence for such effects, such as adaptation to maternal light environments (Galloway & Etterson 2007; Galloway 2005), and transgenerational induction of defences in plants and animals (Agrawal *et al.* 1999), a recent meta-analysis concluded that anticipatory effects were not widespread (Uller *et al.* 2013): In experiments that subjected parents and their offspring to two environments in a fully factorial design, there was only weak evidence that offspring do better when environments are matched rather than mismatched (Uller *et al.* 2013). Although at face value this suggests that evidence for anticipatory parental effects is limited, studies such as those included in the meta-analysis are open to the criticism that the environment parents are subject to is not necessarily a good predictor of the environment offspring would have experienced under natural conditions (Uller *et al.* 2013; Burgess & Marshall 2014)

In species that experience age-related sibling competition, anticipatory parental effects are hypothesised to evolve in order to mediate these age-related effects, and may be related to changes in the size and composition of eggs. Unlike those studies reviewed in Uller *et al.* (2013), parents are assumed to know (rather than predict) where offspring from specific eggs will be placed in any age hierarchy, and thus the competitive environment they may experience. As a consequence, anticipatory parental effects may more easily evolve. For example, Plaistow *et al.* (2007) found that female spider mites increase the size of their eggs as they age, and attributed this to female anticipation of the level of sibling competition later laid eggs will encounter. In birds, egg size (Slagsvold *et al.* 1984) and many egg constituents have been shown to vary, both between females and across the laying sequence of individuals (Williams 2012). These include carotenoids (Royle *et al.* 2001; Blount *et al.* 2002; Török *et al.* 2007; Saino *et al.* 2002), vitamin E (Royle *et al.* 2001) and hormones (Gil 2008). Whilst egg size has downstream consequences on juvenile traits (Krist 2011), hormonal changes in particular have been invoked as mediators of anticipatory parental effects (e.g. Schwabl 1993; Schwabl *et al.* 1997; Lipar *et al.* 1999; Muller & Groothuis 2013), and have also been shown to have downstream effects on multiple aspects of offspring competitiveness (Schwabl 1993, 1996; Gorman & Williams 2005; Groothuis *et al.* 2005; Smiseth *et al.* 2011; Williams 2012).

There is an alternative explanation, however, that changes in egg components across the laying sequence may be the passive outcome of the response of females to their own changing environments, rather than acting in anticipation of the environment offspring will experience. For example, if dietary carotenoids increase over the season whilst eggs are being laid, then the change in eggs may simply reflect the direct effect of carotenoid availability (Török *et al.* 2007). Similarly, changes in female hormones may be seen in preparation for and upon the onset of incubation, leading to different hormonal exposure of eggs (Goldsmith & Williams 1980; Sockman & Schwabl 1999, 2000), reflecting changes

in cumulative exposure to the female's endocrine state. Thus, care is needed to distinguish between anticipatory and passive parental effects.

Previously, we found that prenatal development in blue tits (*Cyanistes caeruleus*) is faster for eggs laid later in the laying sequence, and showed experimentally that this was in part caused by something intrinsic to the eggs at the time of laying (Hadfield *et al.* 2013b). Although consistent with an anticipatory parental effect that acts to reduce the extent of hatching asynchrony, we offered the alternative explanation that it may simply be a result of females being better able to provision later eggs as the amount of resource available to them increases as spring progresses (Hadfield *et al.* 2013b), as there is a rapid change in food availability in this time period (e.g. Dixon 1976). Here, we aim to test this hypothesis by manipulating a female's resource availability during the laying period to see if this accelerates the prenatal development of her offspring. To ensure that any effects detected are the result of changes to the size or composition of the eggs and not due to any effect of the treatment on post-laying behaviour, we cross-fostered half the eggs of each nest on the day they were laid. We are primarily interested in any effects of this treatment on the hatching time of eggs, but also model whether it has any effect on egg weight or hatching success.

The results of our experiment showed little to no effect of the additional resources on prenatal development and other egg characteristics. An alternative explanation is that seasonal changes in the environment are causing laying-order effects on pre-natal development, but in a way that was not be recapitulated in the feeding treatment. Thus, using a larger data set we performed a more general (but correlative) test of whether the rate of pre-natal development varied systematically with three weather variables, which could be drivers of seasonal changes.

Materials and Methods

The experiment was carried out during the springs of 2012 and 2013 on a nest box population of blue tits (*Cyanistes caeruleus*) on the Dalmeny estate, Edinburgh, UK. Nest boxes were placed approximately 30m apart, on two study sites – 180 boxes on Cragie Hill (grid reference NT 156 766) and 45 boxes along the Almond River (NT 179 758). From early in the spring, next boxes were checked systematically to detect nesting and onset of laying. When nest boxes were empty, or had a very small amount of nesting material within them, they were checked every 4 days. When ¿15mm of nesting material was present, boxes were checked every 2 days, and once the nest was lined it was checked every day. This method meant that in all but very few cases, nests were checked on the day in which laying began, and thus a single egg was found.

Cross-foster Design

The study was carried out within an ongoing partial cross-foster design used in this population (for full details see Hadfield *et al.* 2013a). On the day of clutch initiation, nests were randomly allocated into groups of three where possible (or two, four, or five if not), between which eggs were switched. First eggs were then moved within triads, such that the egg from nest A was placed in nest B, that from nest B in nest C, and that from C placed in A. One egg is laid per day, so on alternate days, eggs were either moved between nests, or remained within their own nests, and every egg was weighed and marked. Crossing ceased when one or more nests had a laying pause and resumed when all nests recommenced laying. Daily checks ceased when the female was incubating for the second day in a row, or was found incubating after laying had ceased. The crossing of eggs within a triad stopped when one nest in a given triad finished laying.

Feeding experiment

Within this cross foster design, a feeding experiment was also carried out in order to determine how food availability to parents affects the hatching time of eggs. We used those nests that were in crosses of 3 or more, and each group was assigned randomly to either a control group or one of two feeding treatments. When a group was assigned a feeding treatment, two nests within that group were fed, and the remaining nest (or nests if the group was larger than 3) remained as a control. Nests in the feeding treatments were provided with twenty wax worms (the larvae of the wax moth, *Gralleria mellonella*) per day, which were pinned to narrow tree branches close to the nest box (¡5m) early each morning. On the following day, the number of wax worms consumed was recorded, and any uneaten wax worms were removed. We set up video cameras at some nests to check that the resident birds were taking the food. In some cases other birds were observed taking the wax worms, but this was relatively rare. Blue tits carry out high levels of courtship feeding during the egg laying period (Royama 1966; Krebs 1970; Cramp & Perrins 1993), so we hope that resources from any wax worms taken by the male are passed to feed the female, either directly or indirectly. Nests in the feeding treatments were split into an early group, which were provided with wax worms on days 1 to 4 of the laying sequence, and a late group that received wax worms on days 5 to 8. Both fed nests within a cross-fostering group were given the same treatment. If development of the embryo is resource limited we expect chicks from eggs laid in the treated nests to have more rapid prenatal development if the food provided lifts this constraint. We predict that this effect should be more pronounced in chicks from eggs in the early treatment if resource limitation is more acute at the onset of laying than later in the sequence. Development of eggs takes around 4 days from the onset of rapid yolk development (Haywood 1993), and so eggs 5-8 are expected to be the most affected in the early treatment, and 9 to 12 in the late treatment. During 2012 there were 20 nests in each of the early and late treatment, and 18 in each treatment in 2013, so

158 there were equal numbers in each treatment overall. There were 63 control nests in 2012, and 60 in 2013. In addition, those nests that were not in cross-fostering groups of three
160 or more were never included within the feeding treatment, and so are classed as having no treatment, as they were not true controls. There were 29 such nests in 2012, and 17 in
162 2013. The mean number of wax worms eaten per day in the early treatment was 15.8, and 17.7 per day in the late treatment.

164 The clutch sizes of the nests included in each treatment are shown in Figure 1, along with the distribution of eggs within the laying sequences from each of these treatments. It
166 is worth noting that the clutch size and maximum egg number (a measure for each egg of the days since the first egg was laid in a nest, with the first egg being numbered one) of a
168 nest do not necessarily match due to interruptions in the laying sequence. For example, a female may lay a total of 9 eggs, but may pause in laying, such that eggs were not laid
170 on day 3 and 4 (i.e. there are no eggs three or four), and the maximum egg number is 11 rather than 9. Thus although mean clutch size is 8.26, many nests have several eggs with
172 egg numbers substantially greater than this, even if the total number of eggs in that nest is less than this mean.

174 *Figure 1 here*

Hatching times

176 Nests were checked daily for hatching from 11 days after clutches had been completed. As nests were checked daily, we found chicks within twenty-four hours of the first individual
178 hatching. On this occasion (day 0), the identities of any unhatched eggs were recorded, and

the same was done on the following two visits (day 1 and day 3). No eggs hatched after this point.

Weather

Hourly temperature (°C), rain (ml), and wind speed (knots) measurements were obtained from the Met Office (UK Meteorological Office 1853-Current). Wind and temperature data were from Turnhouse weather station (NT15988 73905, 2.72km from Craigie Hill, and 2.69km from Almond River), whereas rain data were from Gogar Bank (NT17088 71601, 5.21km from Craigie Hill, and 4.27km from Almond River). Hourly data were used to calculate daily mean temperature, daily mean wind speed, and daily total rainfall.

Analysis

Analyses were carried out in R (R Development Core Team 2012), using the package MCMCglmm (Hadfield 2010) to fit Bayesian generalized linear mixed models. For those models which had a binary or ordinal response, residual variances were fixed at 1, as these could not be estimated from the data. For other models a flat improper prior for residual variances was used. For the random effects, parameter expansion was used resulting in scaled $F_{1,1}$ priors on the variances with scale 1000. Fixed effects had independent normal priors with zero mean and large variances (10^8) except in binary/ordinal models. Here, a prior correlation matrix was defined as one in which the fixed effects are identically and independently distributed had the covariates been subject to Gelman's (2008) scaling and centering. The prior correlation matrix was then scaled by six (which in all models was approximately the sum of the variance components) plus $\pi^2/3$ or 1, depending on whether

the logit or probit link was used. Feeding models were run for 130000 iterations, with a burn in of 30000 and a thinning interval of 50, whereas weather models were run for 1300000 iterations, with a burn in of 300000 and a thinning interval of 500 as the chains within these models took much longer to mix well.

Fixed effects are considered significant if the 95% credible intervals did not overlap zero, and pMCMC (twice the posterior probability that the estimate is either negative or positive, depending on which is the smaller probability) is less than 0.05. For groups of fixed effects (e.g. year, treatment) their significance was tested using omnibus Wald tests.

Feeding Experiment: egg-level effects

A series of models were developed to look at the effect of the treatment on individual egg characteristics. The main focus was on hatching time, although we also analysed hatching success, egg weight, and pausing in laying. In all models, clutch size, year, day of clutch initiation (from 1st April) and whether the egg was laid after a pause in the laying sequence or not were fitted as fixed effects. Where necessary, we distinguish between variables measured in the nest-of-origin and nest-of-rearing using the subscripts o and r respectively. The rank_r of the egg (the number of days between it and the final egg in the nest-of-rearing being laid), was fitted as a spline to capture any non-linearity in the relationship induced by incubation behaviour (Hadfield *et al.* 2013b). In all models we excluded eggs that had not been found on the day they were laid, and those from clutches smaller than three eggs (due to early desertion of the clutch). We also excluded three eggs that weighed less than 0.6g, as these were abnormally small (below half the mean egg weight), and do not develop.

For each egg we also included the treatment group of its nest-of-origin (treatment_o) and

its nest-of-rearing (treatment_r) as a fixed factor. Nests that were neither fed nor assigned as controls were included in the analysis as a fourth level in treatment group (coded as 'none'). Generally, these were nests that initiated laying early or late in the season when few nests initiated, so that they could not be assigned to groups of three or more.

Eggs within nests of a given feeding treatment vary in when and how many wax worms were consumed by their mother during their development. For example, the fifth egg in an early-treated nest could be affected by up to 80 wax worms provided over the four consecutive days prior to it being laid, whereas the second egg could only be influenced by up to 20 wax worms on the day prior to it being laid. Blue tits are income breeders, and eggs develop over a period of four days (Haywood 1993), and thus the main eggs expected to be affected by the feeding are those laid around four days after feeding occurs (e.g. egg 5 for extra food given on day 1) if supplementary feeding has a direct effect on eggs. To accommodate this, we included the number of wax worms that were consumed on each day prior to the laying of an egg as multiple predictors. Their effects were modelled using a varying coefficient model (Wood 2006; Hastie & Tibshirani 1993): a type of spline that in this context allows changes in the effect of the predictor (number of wax worms eaten) to be a smooth function of some other covariate (the interval of time between the wax worms being eaten and the egg itself being laid). These models have recently been used in an ecological context (Roberts *et al.* 2015) and in our context can capture any physiological lags that exist between the consumption of food and any effect of that food on the egg. Separate varying coefficient splines were fitted for the early and late groups.

The degree to which the treatment splines improved model performance was assessed using 20-fold cross-validation (Stone 1974, 1977), where models were rerun using data with the k^{th} subset removed. The posterior mean predicted values for the omitted data were obtained with the random nest-of-origin and nest-of rearing effects marginalised using

posterior predictive simulation. Where responses were discrete, the correct classification of the response variable was used as a measure of predictive ability. For continuous responses the coefficient of determination (R^2) was used. Differences between models in their predictive ability were often small, and so we tested whether an increase in predictive ability relative to that of the null model (i.e. one without treatment splines) was greater than expected from Monte Carlo error (i.e. differences were not solely due to random allocation of observations to subsets). This was achieved using repeat-measure ANOVA with subset as a random effect.

Hatching time was analysed in two ways. Firstly, hatching asynchrony was fitted as an ordinal response, which considers the hatching interval of an egg within a clutch (i.e. the hatching day, relative to the first egg in the clutch to hatch, which could be 0, 1 or 2-3). Secondly, as a censored Gaussian response, which fits the time from the final egg in a nest being layed to the hatching of each egg with the censoring points being the day/time of nest visits between which an egg hatched. Eggs were assumed to be laid at 6am, as the exact laying time was not known, but eggs had always been found after this time (Pullen 1946; Perrins 1979). This measure captured differences between nests in incubation behaviour of females (time to commence incubation, and duration of incubation), and within-nests it captures intrinsic differences in hatching time between eggs. Hatching success was analysed as a binary response (hatching/not-hatching) and all abandoned nests were excluded. Egg weight was analysed as a Gaussian response, and the probability of an egg being laid as a binary response (where an egg being laid was given the value 1). These two phenotypes are not dependent on post-laying (and therefore post-crossfostering) effects and so nest-of-rearing terms (both fixed and random) were dropped from the model. In addition, clutch size$_o$ was dropped from the model of pausing, as the two are confounded.

Feeding Experiment: nest-level effects

A second set of models investigated the nest-level effects of the feeding experiment, specifically how it affected clutch size and the onset of incubation. The onset of incubation is taken as the number of days before (negative value) or after (positive value) the last egg was laid that the female was found incubating, or the eggs were found to be warm. Both traits were treated as Gaussian and the models had the same form as above, although treatment was included only as a fixed effect, without spline terms, and egg rank was not included in the model. In addition, only nest-of-origin level terms were retained.

Weather

In addition to the main analyses, we ran the same models as those described above, but also included terms to model how weather (a measure of external conditions) affects egg- and nest-level characteristics. In these analyses we used comparable data collected in the previous two years (Hadfield *et al.* 2013b,a) although egg weight data were unavailable for 2010. Treated nests from 2012-13 were excluded, as were those eggs included in an experiment performed in 2010-2011 (Hadfield *et al.* 2013b). Consequently any terms associated with treatments were dropped from the model. The effect of weather variables (daily mean temperature, daily mean wind speed, and total daily rainfall) were modelled using varying coefficient splines, as was done for the effect of wax worm consumption. Thus each model had three varying coefficient splines within it. Daily weather 50 days prior to, and 40 days after, laying of a given egg was used in these splines, so that both long- and short-term effects of weather might be captured. The sample sizes for each model, along with those eggs and nests that have been excluded are shown in Table 1.

Table 1 here

Results

Feeding Experiment: egg-level effects

Overall, we found little support for any effects of the feeding treatment on hatching time and other egg characteristics. This is seen particularly in the fitting of treatment splines – in all models the treatment splines did not improve the predictive ability of the model, as evaluated using cross-validation (Table 2). Consequently, we report the results of these models with the treatment splines removed in the text and tables. However, in Figure 2, and Figure S2 in the Supporting Information, we present the predictions based on the full model.

Figure 2 here

Table 2 here

The fixed effects for the best model for hatching asynchrony are shown in Table 3. The 95% credible intervals of all fixed treatment effects overlapped zero, and thus there was no significant effect of treatment on asynchrony. There were significant positive effects of clutch size$_r$, and the day of clutch initiation, showing nests are more asynchronous when clutches are larger and laid later in the season. The spline of egg rank (Figure S1a) shows that lower rank eggs hatch later (i.e. late laid eggs hatch late), although this ceases to be the case for eggs of rank > 3 which tend to have equivalent hatching times. Nest-of-origin explained a small amount of variance in hatching asynchrony (0.068 [0.018 - 0.149]), whereas the nest-of-rearing explained a greater proportion of variation in the spread of hatching, 0.562 [0.446 - 0.613].

Table 3 here

318 The fixed effects from the censored Gaussian model of hatching time (from the laying of the last egg in a nest) are summarised in Table 4, again this is the best model (without 320 the treatment splines). Overall, the results are broadly similar to the hatching asynchrony model with all fixed treatment effects overlapping zero and therefore non-significant. There 322 was a significant negative effect of clutch size$_r$, with eggs hatching 0.287 days ([0.127 - 0.445], P¡0.001) faster for each additional egg in a nest, and eggs in later clutches hatched 324 faster by 0.106 days ([0.074 - 0.137], P¡0.001) for each day later in the season. In addition the rank spline (Figure S1b) shows that there is a decrease in time from laying to hatching 326 with increasing rank, although this change is most prominent for those eggs of low rank. The variance explained by the nest-of-origin was small, with an intraclass correlation of 328 0.002 [0.000 - 0.007], but the variance explained by the nest-of-rearing was much larger (0.972 [0.962 - 0.976]) as the censored Gaussian model also includes variation due to 330 differences in incubation behaviour between females. However, the nest-of-origin effect within nest-of-rearing explained more variance (0.079 [0.000 - 0.202]).

332 *Table 4 here*

For the best model of hatching success, the fixed effects are summarised in Table 5 (no 334 treatment splines were in the best model). No significant differences between treatment groups were found, although eggs in none-fed none-control nests were significantly less 336 likely to hatch (-0.863 logits [-1.684 - -0.100] P=0.040). Eggs laid after a pause show a significantly lower probability of hatching than those not laid after a pause. The rank spline 338 (Figure S1c) shows that later laid eggs in a clutch (low rank) are more likely to hatch than

earlier ones, and Figure 2c suggests this is mainly due to first eggs having lower hatching
340 probability. Both the intraclass correlation for nest-of-origin (0.153 [0.000 - 0.264]), and nest-of-rearing (0.167 [0.055 - 0.287]) were reasonably small and of similar magnitude.

342 *Table 5 here*

The fixed effects from the best model of egg weight are summarised in Table 6; this
344 model did not include any treatment splines. The 95% credible intervals of all treatment effects overlapped zero, thus treatment did not have an effect on egg weight. Eggs were
346 significantly smaller (-0.080 g [-0.111 - -0.049] P¡0.001) in 2013 than 2012 and egg weight increased by 0.002 g per day ([0.000 - 0.003], P=0.028) as clutch initiation day increased.
348 There was also a significant effect of being laid after a pause, with eggs laid after an interruption in laying being 0.051 g ([0.043 - 0.059], P¡0.001) heavier. The rank spline
350 (Figure S1d) shows that there is a general decrease of egg weight with increasing rank, implying that late laid eggs tend to be heavier than earlier ones. A large proportion of the
352 variance in egg weight was explained by nest-of-origin (0.800 [0.768 - 0.832]).

Table 6 here

354 The fixed effects from the best model of the probability of an egg being laid are summarised in Table 7 (treatment splines did not improve the model). These results
356 suggest that feeding had no significant effect on the probability that an egg is laid. There is a significant negative effect of the day of clutch initiation, such that pausing occurs more
358 frequently later in the season. The rank spline, Figure S1e, shows a negative effect of egg rank on the probability that an egg is laid, such that interruptions in the laying sequence

are more likely to happen early in a clutch. The intraclass correlation for nest-of-origin was moderate: 0.179 [0.088 - 0.312].

Table 7 here

Feeding Experiment: nest-level effects

The fixed effects from the model of clutch size are summarised in Table 8. Neither treatment had a significant effect on clutch size – early treatment resulted in 0.413 more eggs ([-0.130 - 0.964], P=0.136) than control nests; late treatment resulted in 0.386 more eggs ([-0.197 - 0.947], P=0.170). The difference between early and late treatments overlapped zero (0.026 [-0.640 - 0.676] P=0.917), and the omnibus test showed there was no overall significant effect from the treatments (Wald test χ^2=3.172, P=0.205). There is a significant negative effect of the date on which the clutch started, with females laying 0.073 fewer eggs ([0.049 - 0.095], P¡0.001) with each additional day after April 1st that the clutch started.

Table 8 here

The fixed effects from the model of incubation onset are summarised in Table 9. There was a marginal effect of early treatments on the onset of incubation, with incubation commencing 0.639 days later ([-0.119 - 1.309], P=0.090) than in controls. Although there was no effect of the late treatment, the difference between early and late treatments overlapped zero (0.703 [-0.164 - 1.609] P=0.107). As with clutch size, there was a significant negative effect on the incubation onset of the clutch initiation day – incubation began 0.080

days ([0.048 - 0.114], P¡0.001) earlier, relative to the final egg, with each day after 1st of April that the female commenced laying. Additionally, there is a significant negative effect of clutch size, such that incubation onset advanced by 0.261 days ([0.087 - 0.417], P=0.003) for each additional egg in a clutch.

Table 9 here

Weather

Generally, we found little support for weather having effects on hatching time, and other egg characteristics. In all models, except the censored gaussian model of hatching time and the model of clutch size, the weather splines did not improve the predictive ability of the model as evaluated using cross-validation (Table 10). The results of the null models were qualitatively similar to the feeding treatment models and are presented in the supplementary information. The main difference was support for inter-annual differences in all models, even for those egg characteristics and nest effects for which no significant differences between 2012 and 2013 were found. In addition, the significant effect of eggs laid after a pause having a lower probability of hatching (-0.842 logits [-1.520 - -0.126] P=0.028), were not replicated in the larger data set (-0.306 logits [-1.016 - 0.370] P=0.377). The splines for each weather variable are shown in Figures S3, S4 and S5.

Table 10 here

The best supported model for the censored Gaussian model of hatching time was that with temperature and rain splines included, however the full model had a very similar

classification rate so there was little difference between models with and without wind included (correct classification rate of best model 0.2190; Full model 0.2189; Null model 0.2181). The fixed effects from the best model are shown in Table S3, whilst those for the full and null model are shown in Table S2. The weather spline in Figure S4b shows that there was a positive effect of temperature on hatching time (days from last egg laid in a nest to hatching), up to three weeks prior to the egg being laid, implying that eggs that are developing in the female when conditions are warmer then hatch later. However, the converse appears to be the case post-laying, where there is a negative effect of temperature on hatching time, such that warm conditions after an egg has been laid leads to an acceleration in hatching time. Both rain (Figure S5b) and wind (Figure S3b) prior to laying appear to have little effect on hatching time in the full model, although the effect of rain is supported by cross validation. Year has a significant effect on hatching time in the best model (Wald test on year χ^2=13.651, P=0.003), with the biggest estimated difference of an increase of 1.746 days ([0.721 - 2.675], P¡0.001) to hatching between 2011 and 2012. However, year effects were larger in the null model with an increase of 2.429 days ([2.045 - 2.847], P¡0.001) to hatching between 2011 and 2012 (Wald test on year χ^2=153.13, P¡0.001), implying that year and weather effects may be confounded. Similarly, laydate was not significant in the best model, although it was in the null model. In contrast to the feeding model, clutch size$_r$ did not have a significant effect in the full model, but did in the null model, whereas clutch size$_o$ had a significant positive effect on hatching time in both the full and null model.

Inclusion of splines of temperature and wind improved the predictive ability of the model of clutch size (R^2=0.308; Null model R^2=0.259). The weather splines for wind and temperature are seen in Figures S3f and S4f respectively. Wind appears to have a negative effect on clutch size, such that females lay fewer eggs as mean daily wind speed increases. Similarly, temperature negatively affects clutch size, such that smaller clutches are laid

as daily mean temperature increases. Year has a non-significant effect (Wald test on year χ^2=0.305, P=0.883), although this then appears significant in the null model. Laydate did not have an effect on clutch size, although this was significant and negative in the null model, suggesting that seasonal effect as well as year are confounded with weather in this model.

Discussion

The experiment described above was designed to test whether the increased rate of prenatal development across the laying sequence of blue tits could be directly caused by an increase in the resource availability to the mother at the time of laying. We found little support for supplementary feeding accelerating prenatal development; If anything, there was a positive effect of food consumption on hatching time, i.e. hatching occurred later due to supplementary feeding. Thus, we conclude that the changes in resource availability are not likely to be responsible for the intrinsic acceleration of hatching of late laid eggs observed in Hadfield *et al.* (2013b). We do not see any significant effect of treatment on female behaviour, although the effect size for the fixed effect of treatment on clutch size is reasonable large (increased clutch size by half an egg), suggesting that we may lack the power to estimate this effect.

Our results suggest that differences between eggs across the laying sequence are the result of changes in the female that are independent of either the nutritive or climatic environment. Consequently, these results are consistent with the idea that females are manipulating the developmental rate and consequent hatching time of their offspring independently of their own state. The mechanism by which they achieve this could be through increasing the provision of resources to eggs laid later. Consistent with this idea is the fact that egg size increases over the laying sequence, and larger eggs hatch faster

(Rubolini *et al.* 2005; Hadfield *et al.* 2013b). This is a clear extension of previous work that suggested that larger size of late-laid eggs should benefit late-hatched chicks through the effect on the size at hatching of those chicks (Howe 1976; Clark & Wilson 1981), and thus reducing the effect of hatching asynchrony. Where size also affects hatching time, then size differences may affect the extent, as well as the effect, of hatching asynchrony. In addition, Ferrari *et al.* (2006) and Alquati *et al.* (2007) found that experimental removal of albumen delayed hatching, thus the relative amount of albumen and yolk within an egg may be important in influencing prenatal development. Maternally deposited hormones are a more commonly invoked candidate as a mediator of anticipatory maternal effects. More specifically, androgens generally act to accelerate pre- and post-hatching development (e.g. Lipar & Ketterson 2000; Eising *et al.* 2001), reviewed in von Engelhardt & Groothuis (2011), although contrary results have been found (Sockman & Schwabl 2000; Von Engelhardt *et al.* 2006). Previous work on blue tits, however, suggests there is little variation across the clutch in androgen concentration (Kingma *et al.* 2009); a pattern predicted from interspecific comparisons of initial size differences between first and last hatching chicks and yolk testosterone compensation (Muller & Groothuis 2013). Thus, it seems unlikely that androgens, are driving the differences in prenatal development in this particular case, although other hormones may be at play. Nonetheless, our experiment is unable to differentiate between a passive effect of changes in the females endocrine state and an active anticipatory role if hormones are mediating such effects. It is not known whether females are able to independently control blood plasma and egg hormone concentrations, but most hormones in egg yolks are derived from cells in the developing follicle (Huang *et al.* 1979; Hackl *et al.* 2003; Williams *et al.* 2004; Gil 2008), and so the possibility of independent control exists (Groothuis & Schwabl 2008).

It is also possible that laying order effects are a direct maternal response to changing nutritive conditions, but such an effect might not be seen if providing wax worms failed

to recapitulate such conditions. However, the wax worms provided to the birds should contain sufficient calorific and protein content to lift seasonal resource constraints; twenty wax worms constitute 12kcal and 0.67g protein (Sauter *et al.* 2006), whereas blue tits require 13.8kcal per day (Gibb 1957, winter conditions) and each egg contains about 0.12g of protein (Bourgault *et al.* 2007; Murphy 1994, given a mean egg weight of 1.18g) . Thus, although there is evidence that responses to feeding experiments depend upon the protein content of the food provided (Nager *et al.* 1997; Ramsay & Houston 1997, 1998), we believe our supplementary feeding would lift any constraints imposed by limited protein availability. Furthermore, if changing nutritive conditions were generating changes in hatching time, this would be detected in the models using climatic variables as predictors, which was not seen. In addition to macronutrients, it is possible that birds become less constrained by micronutrients as laying progresses. In particular, blue tits do not use skeletal calcium for egg production, so all calcium must be obtained in the diet (Woodburn & Perrins 1997) mainly through consumption of snail shells, bones and grit (Betts 1955; Graveland & Berends 1997; Reynolds & Perrins 2010). Twenty wax worms contains just 0.230mg calcium, much less than the content of an eggshell (58mg; Graveland & Berends 1997). However, although the wax worms may be limiting in certain resources, the energy they provide may still allow greater time and resources to be spent in obtaining those micronutrients.

Previous feeding experiments lend support to the idea that supplementary feeding can lift constraints associated with breeding, since supplemented birds often show advanced lay dates and increased clutch size (see Christians 2002; Robb *et al.* 2008a). However, these experiments usually commence feeding prior to laying, ranging from several months prior to breeding (e.g. Robb *et al.* 2008b) to a few days (e.g. Ramsay & Houston 1997), and the few that have looked at effects on prenatal development often attribute differences to behavioural changes in the female rather than changes in egg characteristics (e.g. Wiebe &

Bortolotti 1994). In the experiment shown here we failed to find any effect of supplementary food on female behaviour, either through clutch size or incubation. Nevertheless, there is limited evidence that supplementary feeding can alter egg characteristics. Effects on egg size in particular have been investigated, and some increases in response to supplementary food have been found (Wiebe & Bortolotti 1995; Horsfall 1984; Ramsay & Houston 1997), although the results are not always replicated (Nilsson & Svensson 1993; Nager *et al.* 1997), and the majority of studies have failed to find any significant effect of feeding (reviewed in Christians 2002). A recent paper from Ruuskanen *et al.* (2016) carried out a similar experiment to the one shown here, and found increased egg mass in fed nests. However, there appear to be differences between mass of eggs in control and fed treatments prior to feeding, and thus the conclusions from this may be questionable.

In this study we fail to see any major effect of the environment on the prenatal development of chicks, either through the provision of food or through correlations with the weather at the time of laying. Thus it seems that egg characteristics and prenatal development are insensitive to external conditions as experienced by the mother. Consistent with this insensitivity is the high repeatability of egg size (Christians 2002; Williams 2012), and other egg constituents (Postma *et al.* 2014), within females. This suggests that the observed laying-order effects are regulated by the female without regard to her external environment. If the laying-order effects are the result of differential resource provisioning by the female, then we suggest that this is in anticipation of the level of sibling competition the chicks will experience. However, if the laying-order effects are mediated by maternal androgens our results are silent as to whether they are passive by-products of the females endocrinological state or whether they constitute an anticipatory parental effect.

Author Contributions

CET designed the experiment, carried out field work and statistical analysis, and wrote

526 the manuscript. JDH helped in experimental design, carried out field work, designed the statistical analysis, and contributed to writing the manuscript.

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Data Accessibility

536 Data and scripts used for this paper are available on Data Dryad DOI: doi:10.5061/dryad.26h4q.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Figure 1: Egg Rank Splines from the Feeding Analyses

Figure 2: Feeding Splines from the Feeding Analyses

Figure 3: Splines of the effect of Wind from the Weather Analyses

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This manuscript was prepared with the AAS LaTeX macros v5.2.

Figure 4: Splines of the effect of Temperature from the Weather Analyses

Figure 5: Splines of the effect of Rain from the Weather Analyses

Tables 1-9: Results from the models of the effects of Weather on Egg- and Nest characteristics.

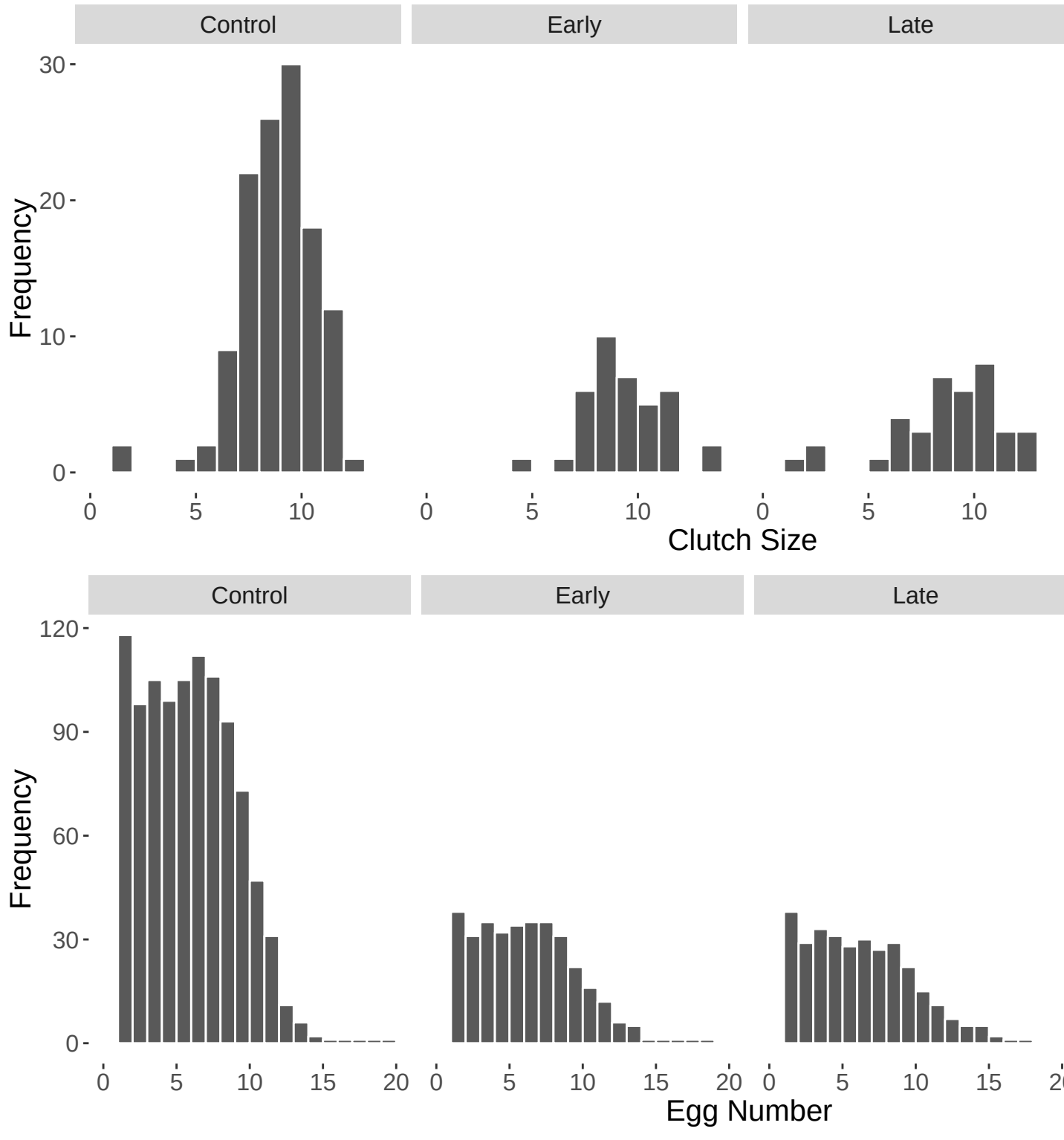

Fig. 1.— The clutch sizes (upper panel) and egg numbers included in the data for each of the treatments. An egg's number is the day on which it was laid (relative to the first egg in the clutch), such that egg 1 is the first egg, and egg 3 is laid 2 days after the first egg. Numbering is irrespective of whether a pause in the laying sequence has occurred, so does

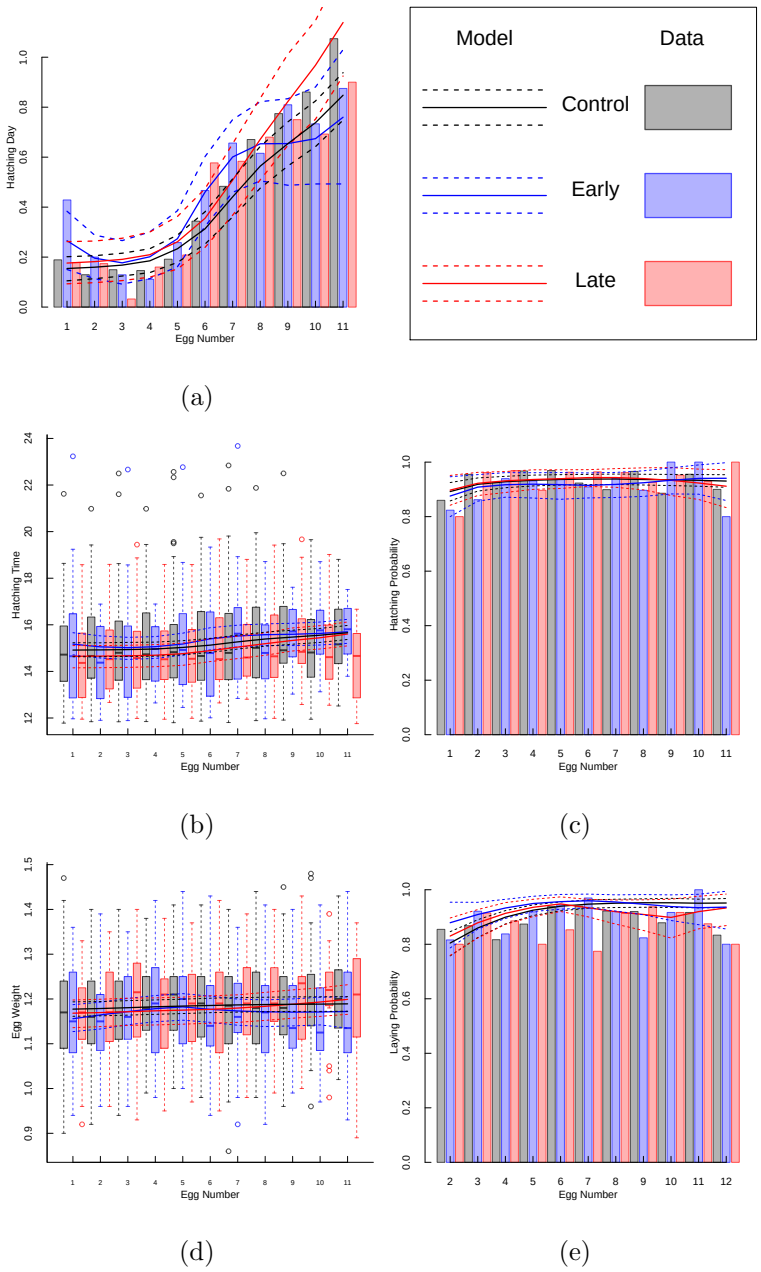

Fig. 2.— The cumulative effect of wax worms eaten on (a) hatching asynchrony within nest, (b) time from laying of the final egg in a nest to hatching, (c) probability of hatching, (d) weight of eggs, and (e) the probability that an egg is laid. Boxplots and barplots show the raw data, split by treatment, together with predictions (solid lines) and 95% credible intervals (dashed lines). Predictions were made holding all predictors at their mean value except rank and the number of wax worms eaten for which we use the mean for each egg-number/treatment combination.

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Table 1: Sample sizes (n), the number of eggs that are excluded, and the number of nests that those eggs are found in, for each model

	Feeding				**Weather**			
	Included		Excluded		Included		Excluded	
	Eggs	Nests	Eggs	Nests	Eggs	Nests	Eggs	Nests
Ordinal Hatching	1649	214	67	3	3076	426	775	39
Censored Gaussian Hatching	1649	214	67	3	3076	426	775	39
Hatching Success	1825	215	49	2	3425	428	829	37
Weight	1979	235	51	12	2754	319	730	91
Pausing	1739	235	0	0	3259	431	794	93
Clutch Size		229		18		409		115
Onset of Incubation		210		24		391		104

Table 2: Mean predictive power from 20-fold cross validation of feeding experiment models, comparing the full model (all fixed and random effects included, along with splines of the effects of the feeding experiment) with those that drop the splines for each of the early and late treatments, and the null model in which both are dropped (but all other fixed and random effects are retained). Ordinal and Censored Gaussian are the two models of hatching time.. For weight, predictive power is measured as an R^2 value, and for other models it is the rate of correct classification. In all cases the predictions are marginal with respect to nest_o and nest_r (where appropriate). The final column is the probability that the estimated predictive power of the best model (in bold) exceeds that of the null model more than would be expected from Monte Carlo error alone.

	Full Model	Early Spline Dropped	Late Spline Dropped	Both Dropped	Pr(¿W)
Ordinal	0.616	0.615	**0.616**	0.615	0.1240
Censored Gaussian	0.178	0.178	**0.178**	0.177	0.0830
Hatching Success	0.842	0.842	0.842	**0.843**	
Weight	0.094	0.095	0.096	**0.097**	
Laying Success	0.793	0.793	**0.794**	0.793	0.5090

Table 3: Summary of the fixed effects from an ordinal model of hatching asynchrony (day of hatching relative to the first day of hatching within the nest). These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	-6.067	-8.539	-3.654	**¡0.001**	
Treatment_r.None	0.288	-0.442	1.120	0.464	
Treatment_r.Early	-0.200	-1.150	0.675	0.646	0.223
Treatment_r.Late	0.731	-0.210	1.588	0.121	
Treatment_o.Early	0.585	-0.057	1.285	0.101	0.232
Treatment_o.Late	-0.082	-0.789	0.586	0.803	
Clutch size_r	0.284	0.105	0.495	**0.002**	
Clutch size_o	0.083	-0.048	0.212	0.221	
Year.2013	-0.389	-1.156	0.403	0.308	
Laydate_r	0.052	0.014	0.091	**0.007**	
After pause	0.365	-0.131	0.867	0.163	

Table 4: Summary of the fixed effects from a censored Gaussian model on the time (in days) from laying to hatching of eggs. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	21.347	19.327	23.197	**¡0.001**	
Treatment_r.None	-0.430	-1.106	0.189	0.193	
Treatment_r.Early	0.079	-0.675	0.711	0.822	0.663
Treatment_r.Late	-0.277	-0.978	0.377	0.438	
Treatment_o.Early	0.101	-0.018	0.219	0.107	0.264
Treatment_o.Late	0.015	-0.118	0.136	0.818	
Clutch size_r	-0.287	-0.445	-0.127	**¡0.001**	
Clutch size_o	0.018	-0.005	0.040	0.111	
Year.2013	-0.358	-0.972	0.337	0.295	
Laydate_r	-0.106	-0.137	-0.074	**¡0.001**	
After pause	0.049	-0.038	0.146	0.322	

Table 5: Summary of the fixed effects from a model of hatching success of eggs. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	0.331	-2.086	3.032	0.809	
$Treatment_r$.None	-0.863	-1.684	-0.100	**0.040**	
$Treatment_r$.Early	-0.490	-1.557	0.499	0.349	0.56
$Treatment_r$.Late	0.258	-0.794	1.319	0.627	
$Treatment_o$.Early	0.149	-0.830	1.195	0.778	0.918
$Treatment_o$.Late	-0.155	-1.162	0.899	0.784	
$Clutch\ size_r$	0.150	-0.050	0.365	0.160	
$Clutch\ size_o$	0.133	-0.024	0.318	0.116	
Year.2013	-0.630	-1.499	0.128	0.125	
$Laydate_r$	0.034	-0.003	0.076	0.089	
After pause	-0.842	-1.520	-0.126	**0.028**	

Table 6: Summary of the fixed effects from a model of egg weight. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	1.160	1.059	1.251	**¡0.001**	
$Treatment_o$.None	-0.005	-0.035	0.027	0.728	
$Treatment_o$.Early	-0.012	-0.045	0.020	0.467	0.766
$Treatment_o$.Late	-0.004	-0.037	0.031	0.771	
$Clutch\ size_o$	-0.001	-0.009	0.007	0.896	
Year.2013	-0.080	-0.111	-0.049	**¡0.001**	
Laydate	0.002	0.000	0.003	**0.028**	
After pause	0.051	0.043	0.059	**¡0.001**	

Table 7: Summary of the fixed effects from a model of the probability that an egg is laid. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	4.469	3.466	5.530	**¡0.001**	
$Treatment_o$.None	-0.530	-1.232	0.082	0.094	
$Treatment_o$.Early	0.394	-0.330	1.116	0.286	0.504
$Treatment_o$.Late	-0.091	-0.782	0.603	0.820	
Year.2013	-0.283	-0.987	0.379	0.399	
Laydate	-0.039	-0.069	-0.004	**0.024**	

Table 8: Summary of the fixed effects from a gaussian model of clutch size. P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	10.828	10.092	11.564	**¡0.001**	
Treatment$_o$.None	-0.112	-0.620	0.467	0.689	
Treatment$_o$.Early	0.413	-0.130	0.964	0.136	0.205
Treatment$_o$.Late	0.386	-0.197	0.947	0.170	
Laydate	-0.073	-0.095	-0.049	**¡0.001**	
Year.2013	0.232	-0.312	0.692	0.402	

Table 9: Summary of the fixed effects from a gaussian model of the onset of incubation relative to the date on which the last egg in a nest was laid. P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(¿W)
Intercept	6.185	4.018	8.138	**¡0.001**	
$Treatment_o$.None	-0.154	-0.820	0.519	0.687	
$Treatment_o$.Early	0.639	-0.119	1.309	0.090	0.187
$Treatment_o$.Late	-0.064	-0.731	0.715	0.838	
Laydate	-0.080	-0.114	-0.048	**¡0.001**	
Year.2013	-0.371	-1.002	0.311	0.298	
Clutch $size_o$	-0.261	-0.417	-0.087	**0.003**	

Table 10: Mean predictive power from 20-fold cross validation of weather models, comparing the full model (all fixed and random effects included, and splines of the effects of temperature, rain and wind) with those with each of the individual weather splines dropped, and the null model in which all three weather splines are dropped (but all other fixed and random effects are retained). Ordinal and Censored Gaussian are the two models of hatching time. For weight, predictive power is measured as an R^2 value, and for other models it is the rate of correct classification. All are run with nest_o and nest_r (where appropriate) marginalised. The final column is the probability that the predictive power of the best model (in bold) exceeds that of the null model more than would be expected from Monte Carlo error alone.

	Full Model	Wind Spline Dropped	Temperature Spline Dropped	Rain Spline Dropped	All Dropped	Pr(¿W)
Ordinal	0.574	0.592	0.578	0.569	**0.593**	
Censored Gaussian	0.219	**0.219**	0.219	0.218	0.218	**0.0180**
Hatching Success	0.831	**0.831**	0.831	0.831	0.830	0.0730
Weight	**0.066**	0.062	0.063	0.064	0.063	0.4000
Pausing	0.868	**0.869**	0.868	0.868	0.868	0.1990
Clutch Size	0.303	0.305	0.271	**0.308**	0.259	**0.0020**
Incubation	0.243	0.247	0.248	0.249	**0.256**	